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Figures and figure supplements

The quantitative architecture of centromeric chromatin

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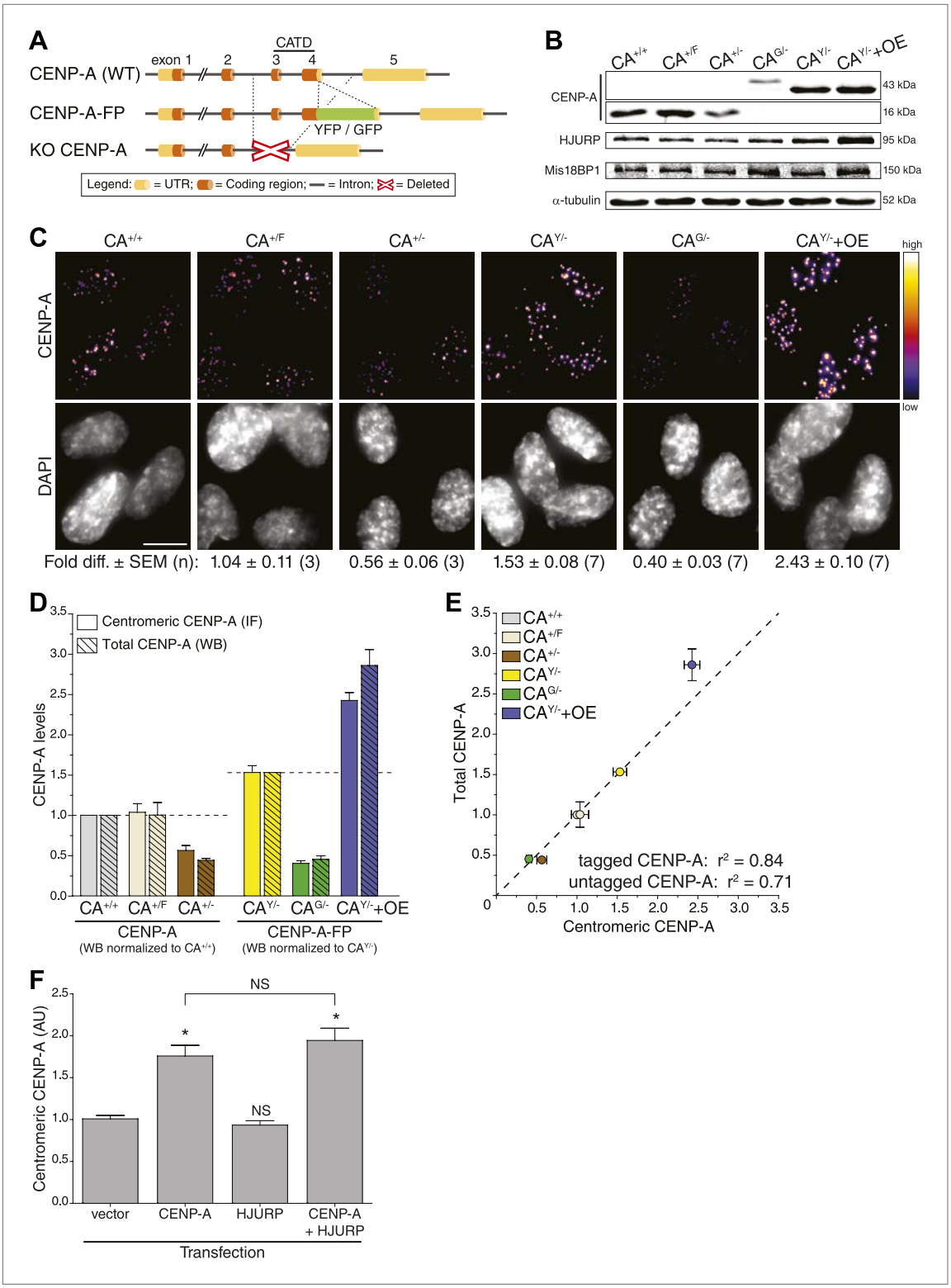

Figure 1. CENP-A levels are regulated by mass-action. (**A**) Schematic of gene-targeting strategy that allowed for the creation of CENP-A knockout and fluorescent knock-in alleles. The region encoding the essential CENP-A targeting domain (CATD, ***Black et al., 2007***) is indicated. (**B**) Quantitative immunoblots of CENP-A, HJURP, and Mis18BP1 in differentially targeted RPE cell lines. α-tubulin is used as a loading control. (**C**) Immunofluorescence images of same cell lines as in **B**. CENP-A intensity is represented in a heat map as indicated on the right. The fold difference ± SEM (n is biological

Figure 1. Continued on next page

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replicates) compared to wild-type RPE cells is indicated below. Scale bar: 10 μm. Note that in contrast to quantification of immunoblots, immunofluoresce detection of untagged and tagged CENP-A is directly comparable. (**D**) Quantification of centromeric CENP-A levels (from **C**) by immunofluorescence (IF) and total CENP-A levels (n = 4–9 independent experiments as in **B**) by western blot (WB). All cell lines expressing untagged CENP-A are normalized to $CA^{+/+}$ while those expressing tagged CENP-A are normalized to the centromeric $CA^{Y/-}$ levels measured in **C**, as indicated by dashed lines. (**E**) Correlation of centromeric and total cellular CENP-A levels as measured in **D**. Dashed line represents a predicted directly proportional relationship with indicated correlation coefficients. Throughout, the average ± SEM is indicated. (**F**) Quantification of centromeric CENP-A levels in synchronized HeLa cells (based on anti-CENP-A staining) within a single cell cycle after transient transfection of indicated proteins. Asterisk indicates statistically significant increase compared to control or indicated transfections (one-tailed *t* test; $p<0.05$; n = 3); NS indicates no significant increase. Average ± SEM of three independent experiments is shown.

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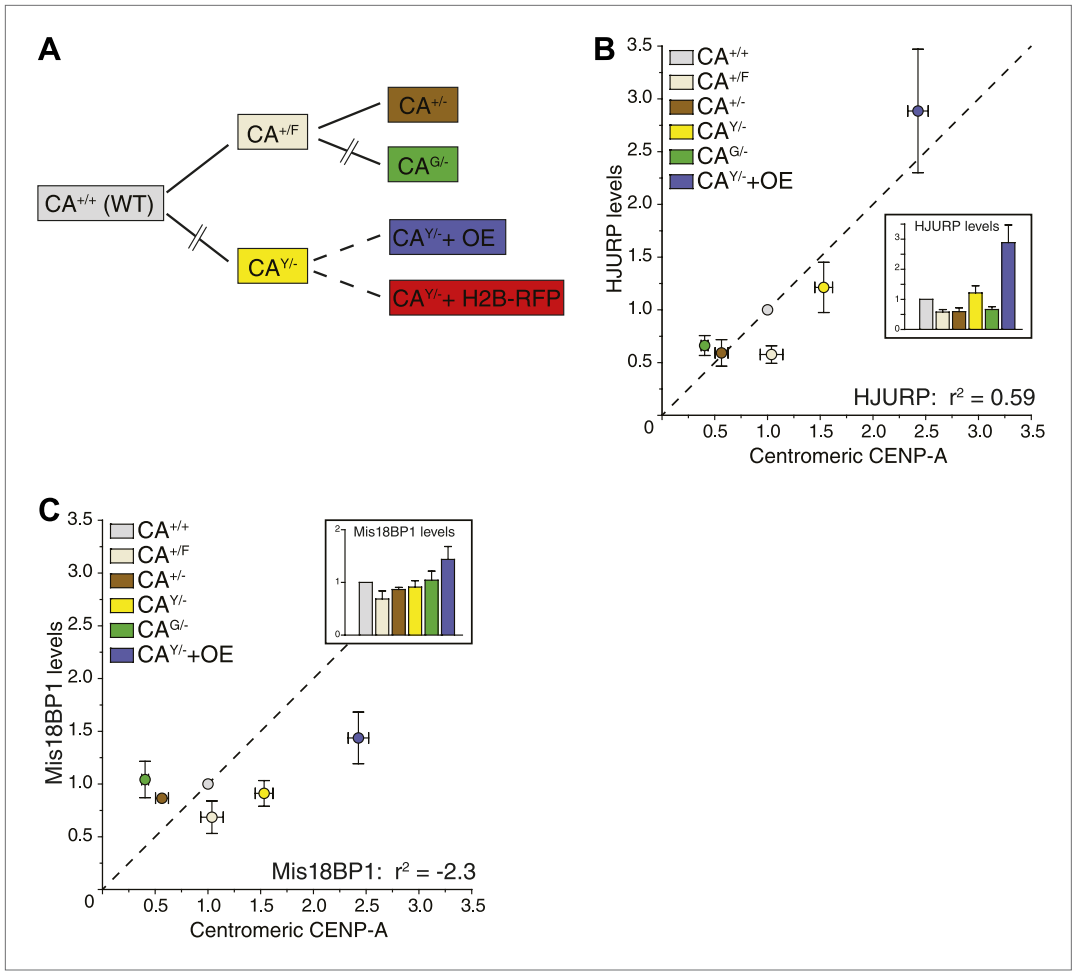

Figure 1—figure supplement 1. CENP-A expression is the rate limiting factor for centromeric CENP-A levels.

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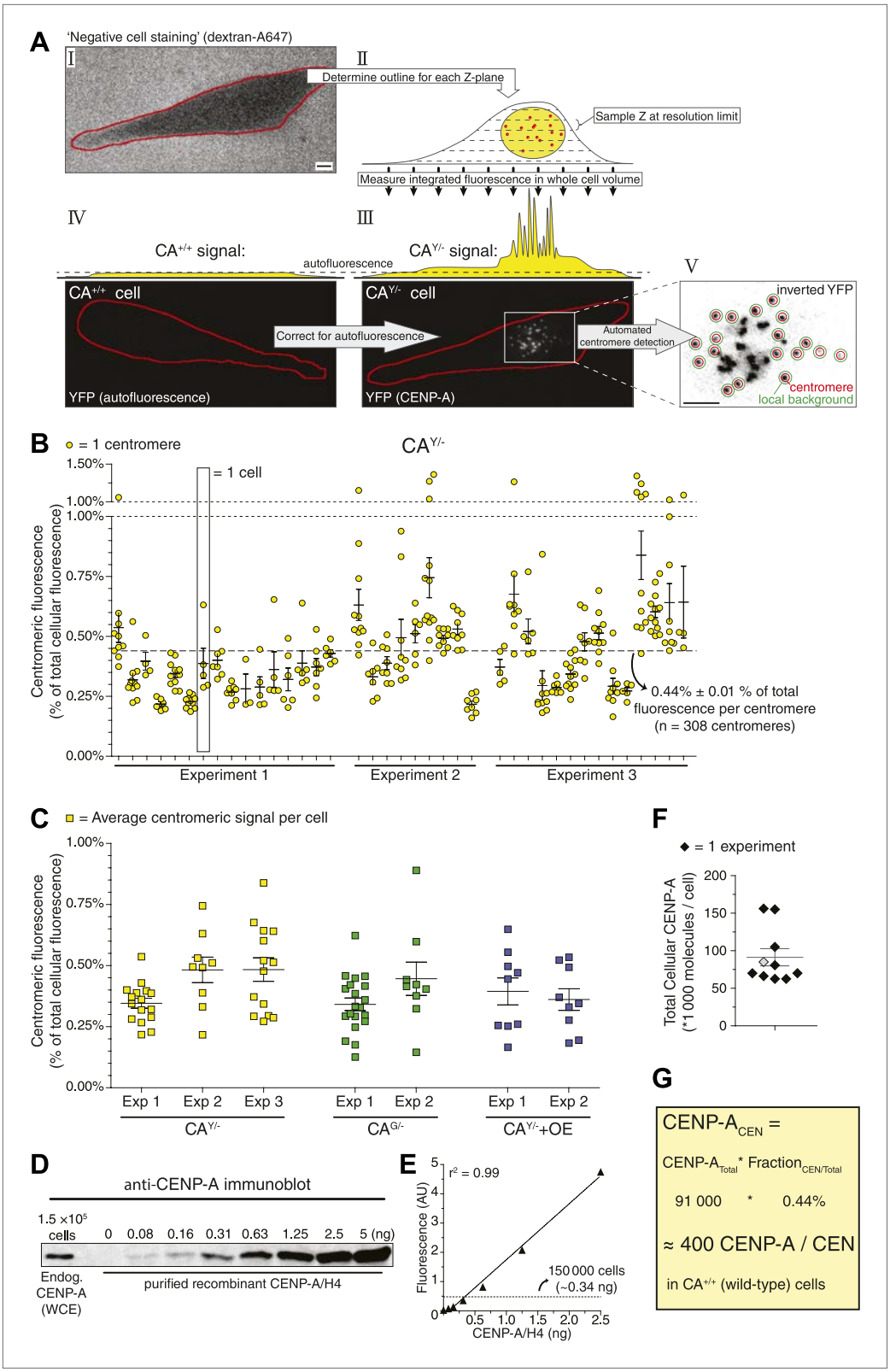

Figure 2. Human centromeres contain 400 molecules of CENP-A. (**A**) Schematic outline of strategy allowing for the quantification of the centromeric fraction of CENP-A compared to the total cellular pool. Scale bars: 5 μm. (**B**) Quantification of the centromeric fraction of CENP-A in $CA^{Y/-}$ cells. Each circle represents one centromere;

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circles on the same column are individual centromeres from the same cell. Dashed line indicates average of all centromeres. (**C**) Quantification of the centromeric fraction of CENP-A in indicated cell lines. Each square represents the average centromeric signal from one cell; squares on the same column are individual cells from the same experiment (Exp). ***Figure 2—figure supplement 2*** shows quantification of individual centromeres in $CA^{G/-}$ and $CA^{Y/-}$+OE cells. (**D**) Representative quantitative immunoblot of purified recombinant CENP-A and endogenous CENP-A from whole cell extracts (WCE). (**E**) Quantification of **D**. Solid line represents the best fit linear regression. Dashed line represents the amount of CENP-A from 150,000 cells. (**F**) Quantification of the total cellular CENP-A copy number. Each diamond represents one replicate experiment; measurement from **E** is indicated as a gray diamond. (**G**) Calculation of average CENP-A copy number per centromere (CEN) in wild-type RPE cells. Throughout, the average ± SEM is indicated.

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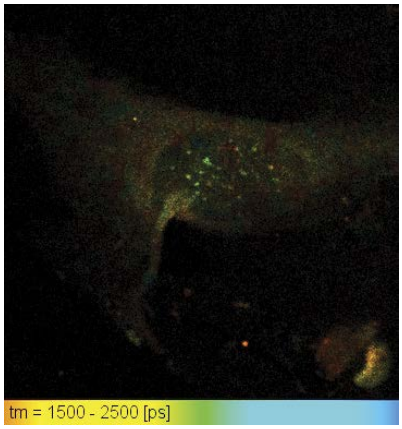

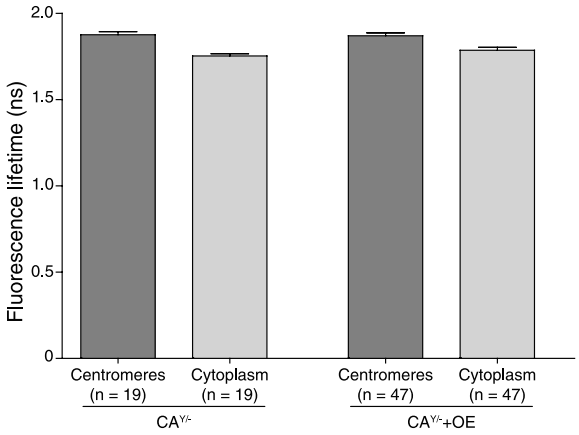

Figure 2—figure supplement 1. Representative fluorescence lifetime imaging (FLIM) micrograph of a CENP-A-YFP expressing cell (left) and quantification of indicated cellular regions (right).

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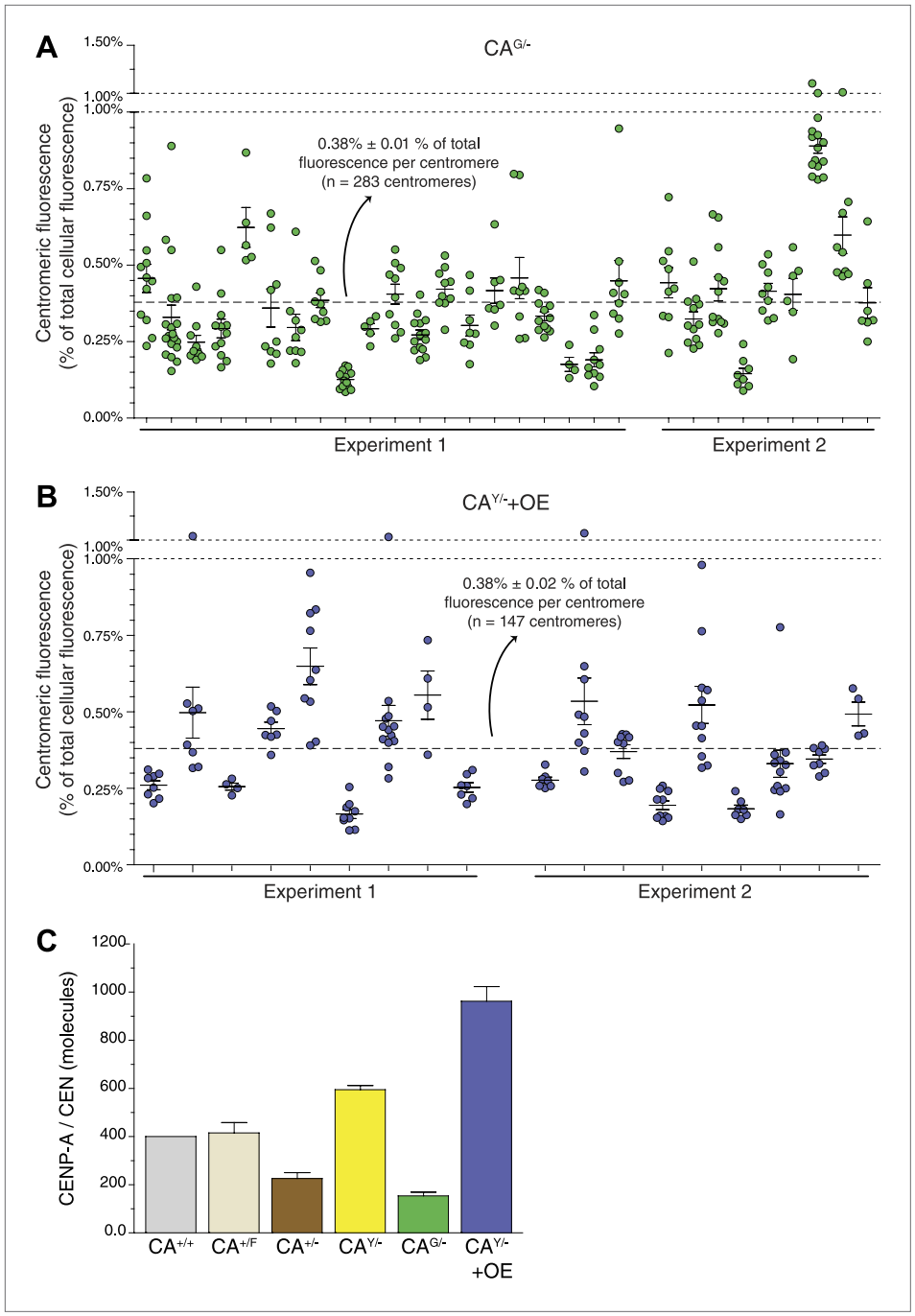

Figure 2—figure supplement 2. Measurements of individual centromeres and CENP-A levels for different cell lines.

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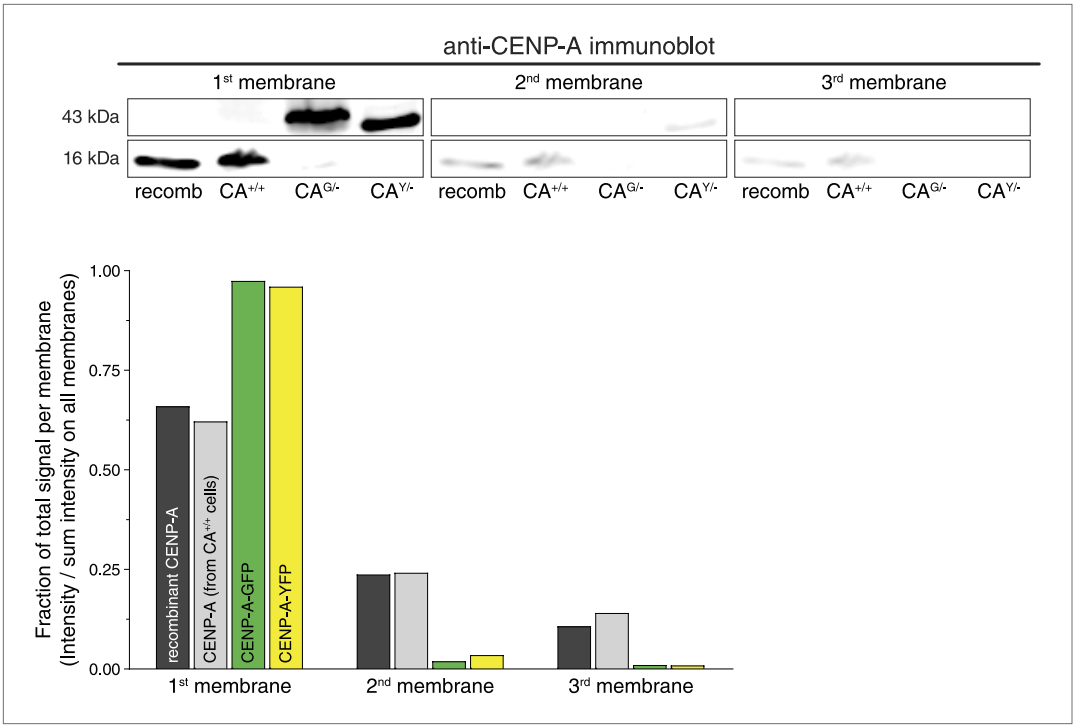

Figure 2—figure supplement 3. Transfer efficiency of recombinant and cellular CENP-A.
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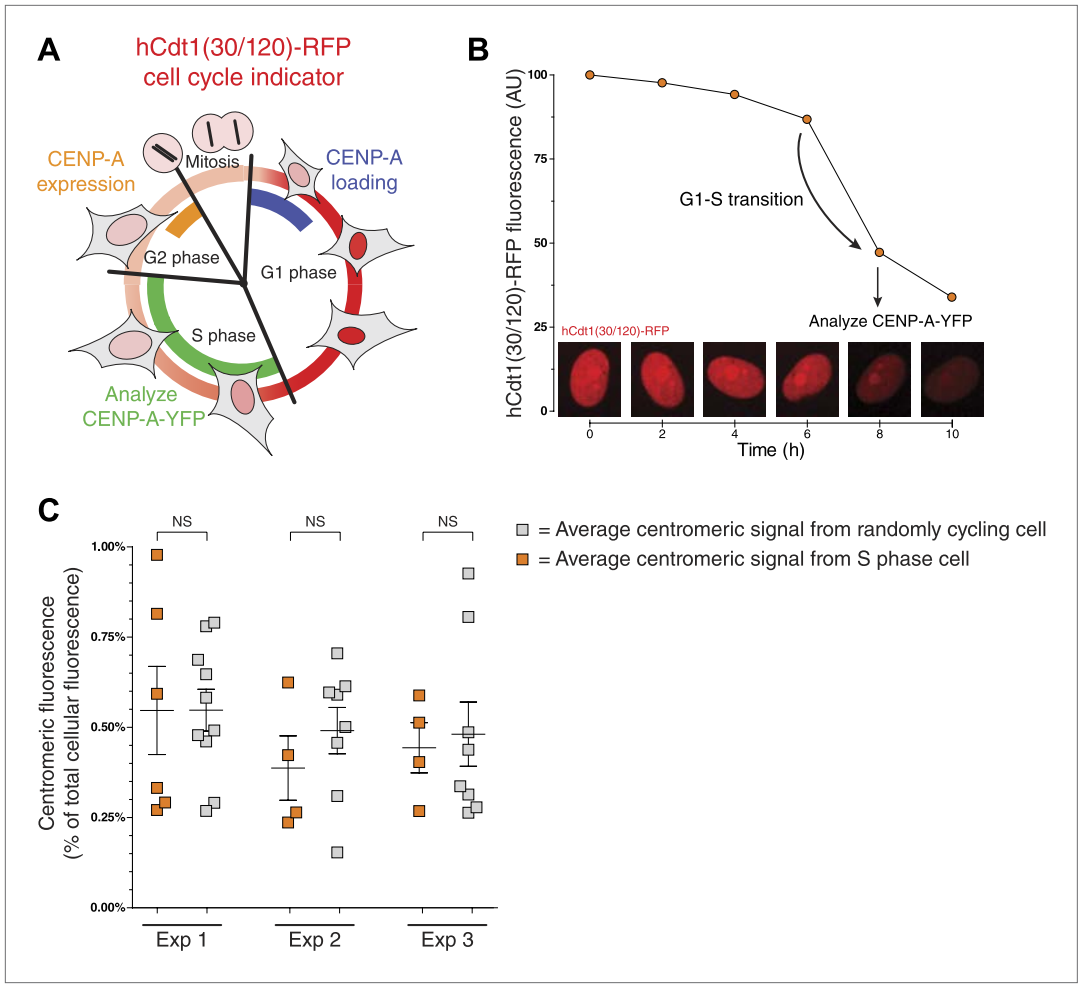

Figure 3. Centromeric CENP-A levels are equivalent between S phase and randomly cycling cells. (**A**) Cartoon depicting changes in cell morphology and nuclear levels of hCdt1(30/120)-RFP (in red) throughout the cell cycle (***Sakaue-Sawano et al., 2008***). Approximate timing of CENP-A expression (***Shelby et al., 2000***) and centromeric loading (***Jansen et al., 2007***) are indicated in orange and blue, respectively. The stage at which cells were analyzed to measure the centromeric fraction of CENP-A is indicated in green. (**B**) An example trace of a cell entering S phase (indicated by a sudden decrease in RFP levels) is shown. The centromeric fraction of CENP-A was measured at this point as outlined in ***Figure 2A***. Peak expression is normalized to 100 and background fluorescence to 0. Micrographs of hCdt-1(30/120)-RFP at indicated timepoints are shown below. (**C**) As in ***Figure 2C***. Orange squares represent cells that have passed the G1-S transition point, as indicated by decreasing levels of hCdt-1(30/120)-RFP. Gray squares represent randomly cycling cells. No statistically significant differences (NS) were observed between randomly cycling cells and S phase cells.

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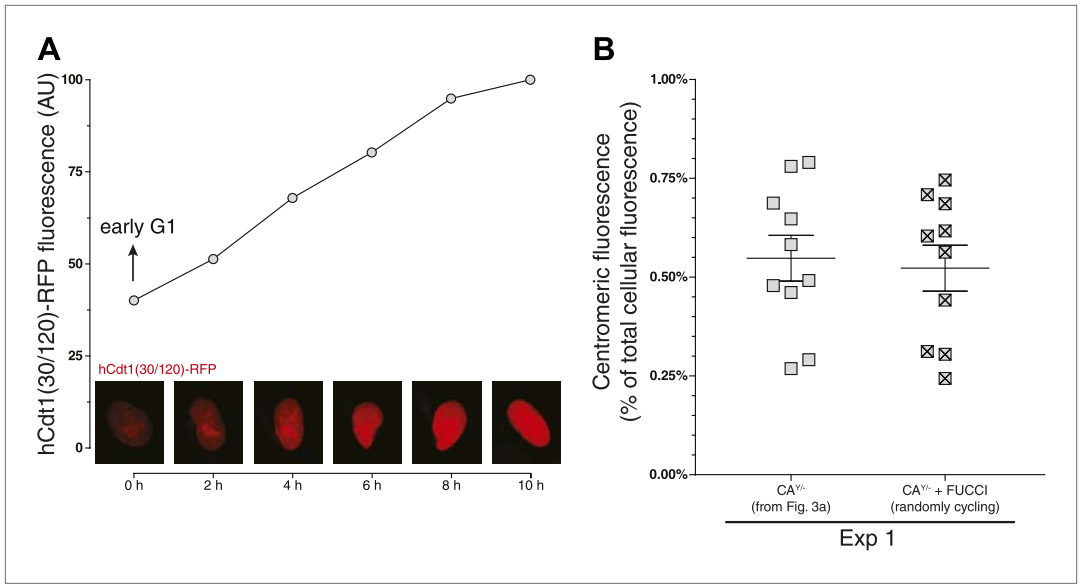

Figure 3—figure supplement 1. hCdt-1(30/120)-RFP expression allows for accurate determination of cell cycle stages and measurements of centromeric CENP-A ratios.

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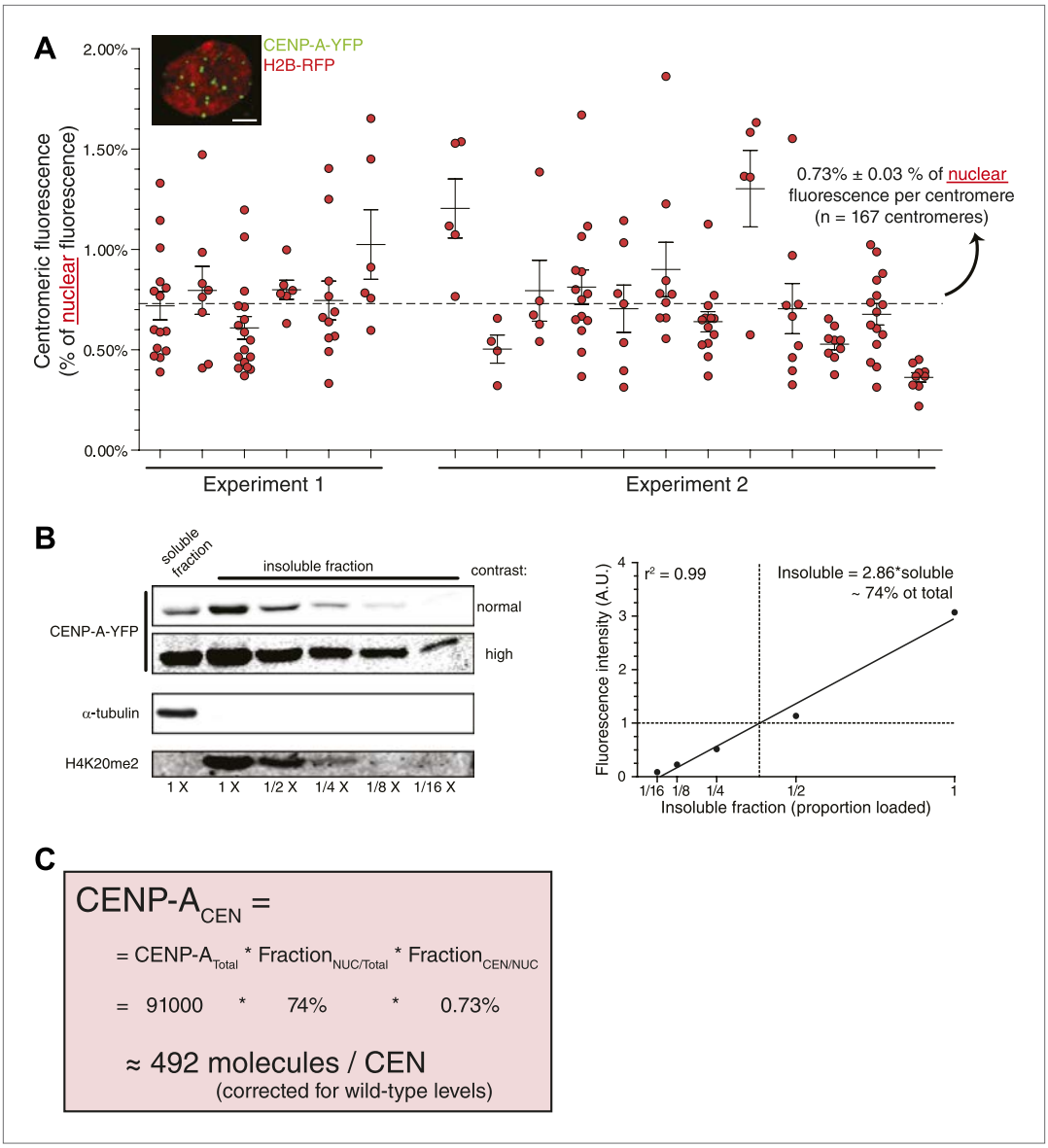

Figure 4. Measurement of nuclear CENP-A confirms centromeric copy number. (**A**) As in ***Figure 2B***, except that the centromeric fraction compared to total nuclear pool is indicated. Inset shows a representative image of a $CA^{Y/-}$+H2B-RFP cell (scale bar: 2.5 μm). (**B**) Quantitative immunoblot showing the soluble fraction and a dilution series from the insoluble fraction of CENP-A-YFP in $CA^{Y/-}$+H2B-RFP cells (left). Tubulin is used as a marker for the soluble fraction and H4K20me2 (exclusively found in chromatin, ***Karachentsev et al., 2007***) for the insoluble fraction. Quantification of insoluble fraction of CENP-A is shown to the right. (**C**) Calculation of the average CENP-A copy number per centromere (CEN) in wild-type RPE cells, based on results from $CA^{Y/-}$+H2B-RFP cells.

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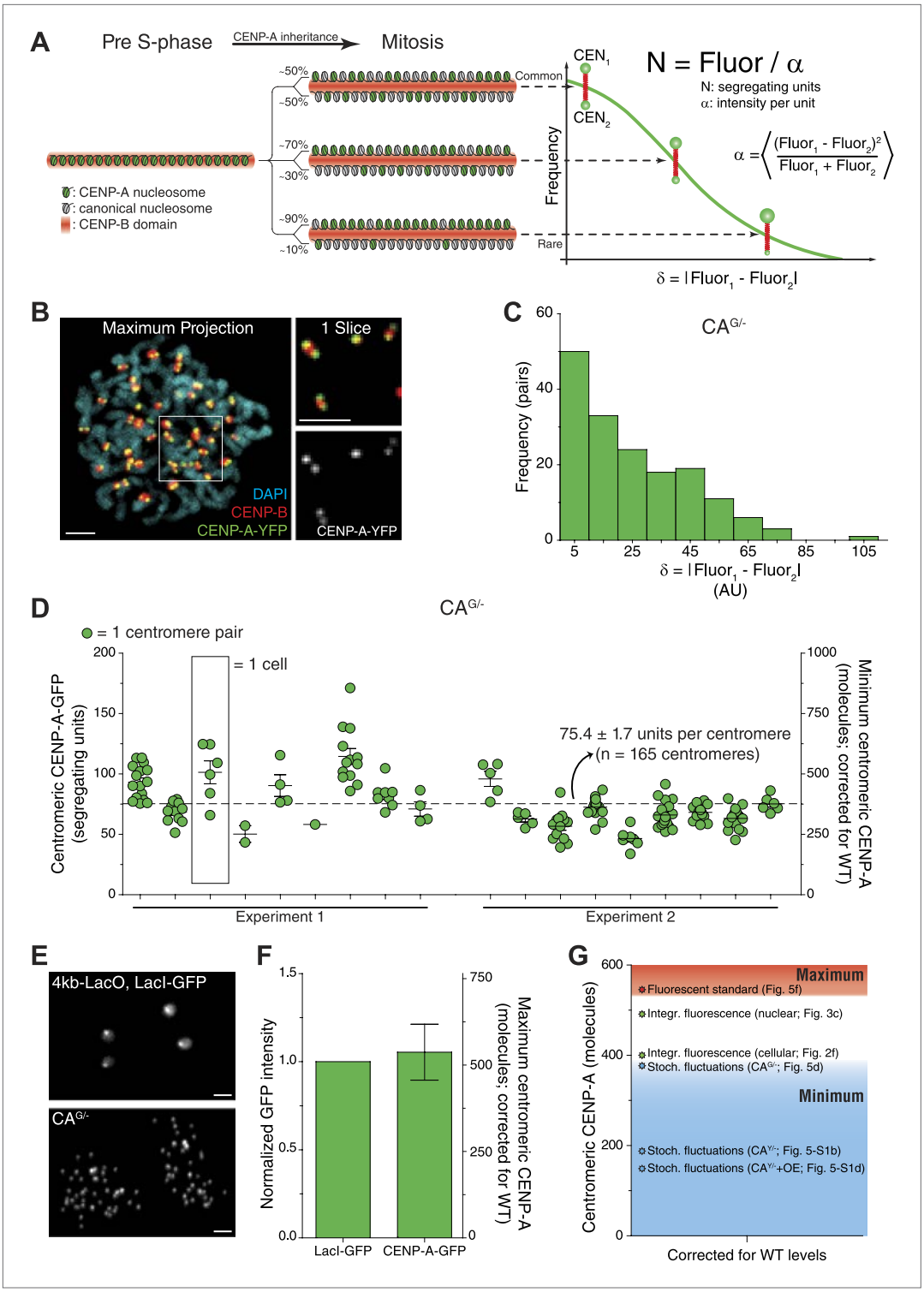

Figure 5. Independent quantification methods confirm centromeric CENP-A copy number. (**A**) Stochastic fluctuation method: cartoon depicting inheritance and random redistribution of parental CENP-A nucleosomes onto sister chromatids during DNA replication. A hypothetical distribution of the absolute difference between the two sister centromeres, as well as the formula for calculating the fluorescence intensity per segregating unit (α) are indicated on the right. (**B**) Representative image of mitotic CENP-A-YFP expressing cell. CENP-B staining allows for identification of sister centromeres. Blowup to the right represents a single slice of the boxed region showing that CENP-B is located in between the CENP-A spots of sister centromeres. (**C**) Frequency distribution of the difference between

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CENP-A-GFP intensity of sister centromeres in $CA^{G/-}$ cells. (**D**) Quantification of centromeric CENP-A-GFP based on the stochastic fluctuation method. Each circle represents one centromere; circles on the same column are individual centromeres from the same cell. Left y-axis indicates segregating CENP-A-GFP units in $CA^{G/-}$ cells; right y-axis shows the conversion to minimum number of centromeric CENP-A molecules in $CA^{+/+}$ (WT) cells. (**E**) Fluorescent standard method: representative fluorescence images of 4kb-LacO, LacI-GFP *S. cerevisiae* and human $CA^{G/-}$ cells. (**F**) Quantification of fluorescence signals of LacI-GFP and CENP-A-GFP spots (n = 2 biological replicates). The left y-axis indicates the fluorescence intensity normalized to LacI-GFP; the right y-axis shows the conversion to maximum number of centromeric CENP-A molecules in wild-type cells. (**G**) Comparison of independent measurements for the centromeric CENP-A copy number (corrected for $CA^{+/+}$ levels; Stoch. fluctuations = stochastic fluctuation method [***Figure 5A***]; Integr. fluorescence = integrated fluorescence method [***Figure 2A***]). Levels from all strategies are corrected for $CA^{+/+}$ (WT) levels. Throughout, the average ± SEM and scale bars of 2.5 μm are indicated.

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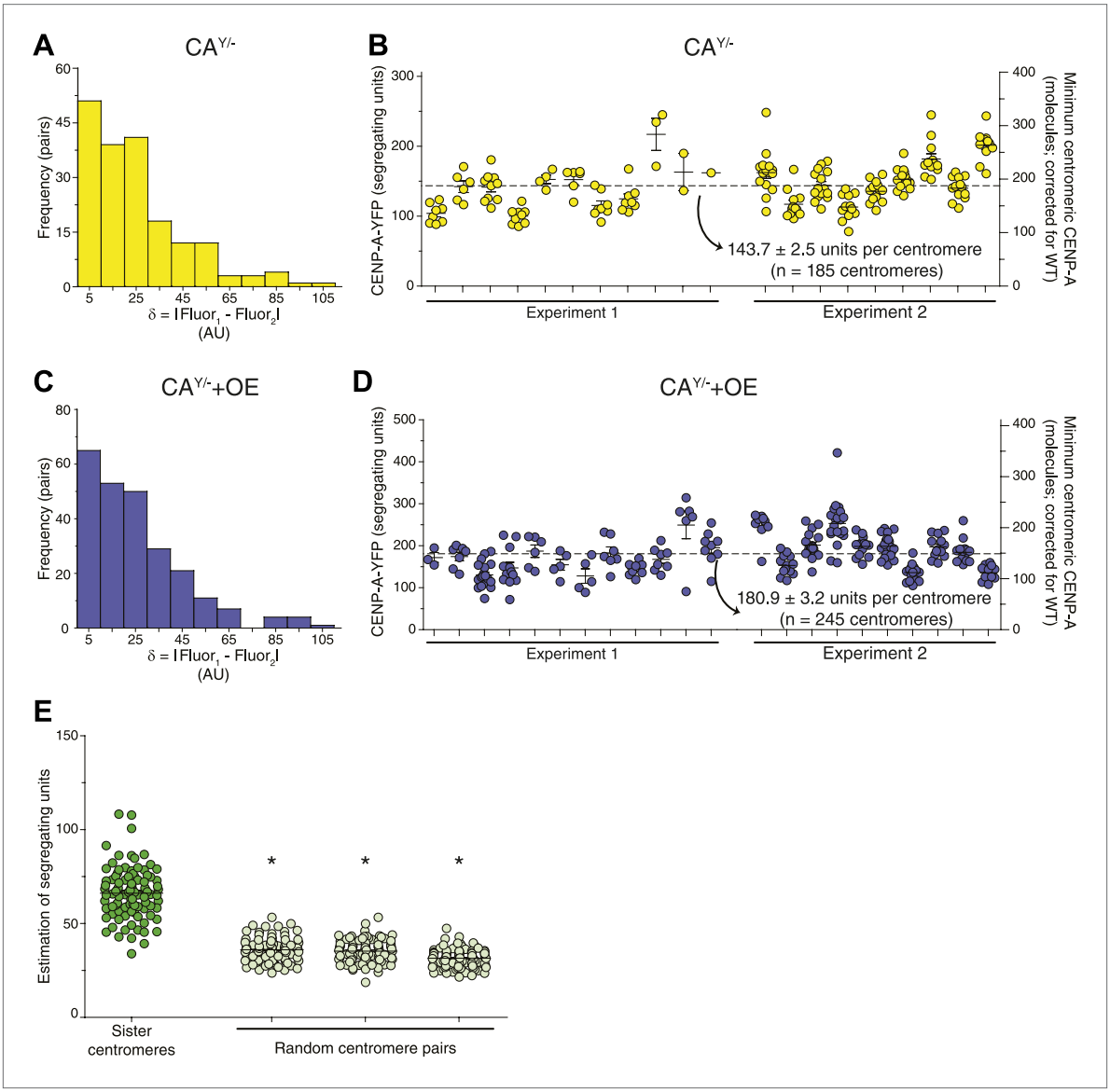

Figure 5—figure supplement 1. Stochastic fluctuations of CENP-A segregation allows for copy number measurements.

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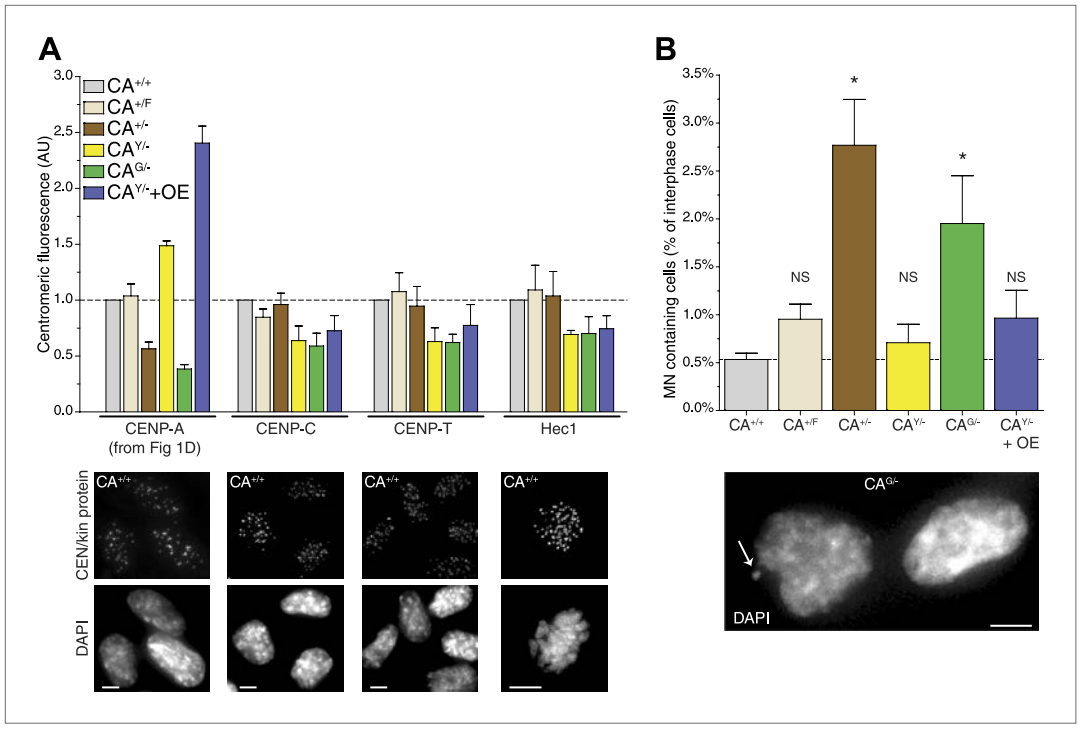

Figure 6. Reduction of CENP-A leads to a CENP-C, CENP-T, and Hec1 independent increase in micronuclei. (**A**) Quantification of centromeric CENP-A (from ***Figure 1***), CENP-C, CENP-T, and Hec1 levels for indicated cell lines; n = 4 independent experiments in each case. Note that cell lines carrying tagged CENP-A have a slight, yet non-significant impairment in recruiting CENP-C, CENP-T, and Hec1. However, this does not correlate with the CENP-A levels themselves. Below, representative images of indicated antibody staining from $CA^{+/+}$ cells are shown. Representative images from all cell lines can be found in ***Figure 6—figure supplement 1***. (**B**) Quantification of the fraction of cells containing micronuclei (MN) for indicated cell lines. Asterisk indicates statistically significant increase compared to wild-type (paired *t* test; $p<0.05$; n = 3–4 independent experiments [500–3000 cells per experiment per cell line]); NS indicates no significant difference. Throughout, the average ± SEM is indicated and dashed lines represent wild-type levels. Scale bars: 5 μm.

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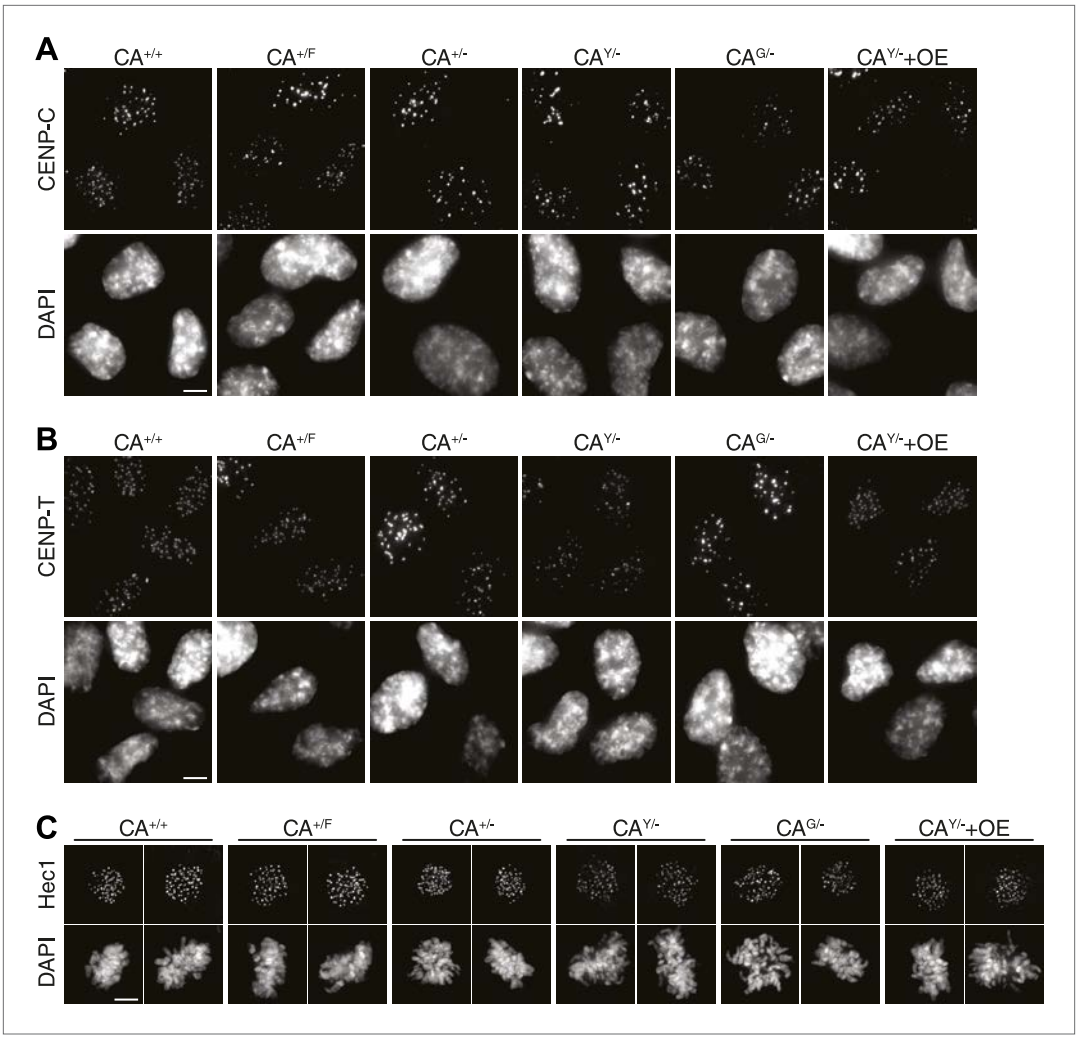

Figure 6—figure supplement 1. Representative images for quantifications in ***Figure 6B***.

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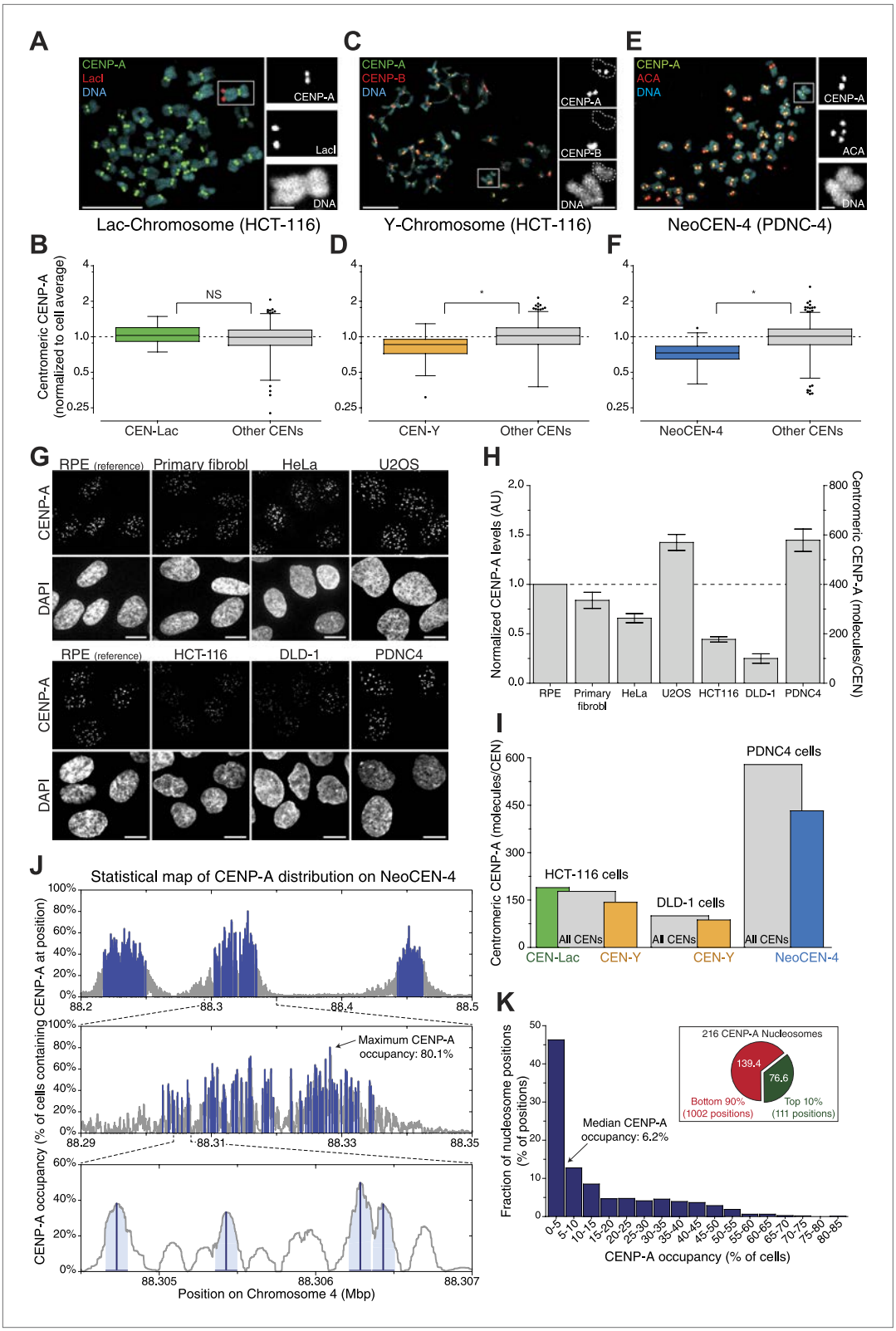

Figure 7. Centromere and cell specific distribution of CENP-A. (**A**, **C**, **E**) Representative micrograph of mitotic spreads for LacI-GFP::LacO expressing HCT-116 cells (**A**); wild-type HCT-116 cells (**C**); and PDNC-4 cells (**E**). Blowups show the chromosome containing the integrated Lac-array (**A**); the Y-chromosome (outline indicated; CENP-B negative) as well as an autosome (CENP-B positive) (**C**); and the neocentric chromosome 4, containing

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2 pairs of ACA spots (staining both CENP-A and CENP-B), but only 1 pair of CENP-A spots (**E**). (**B**, **D**, **F**) Quantification of CENP-A levels on the centromere of the chromosome containing the Lac-array (CEN-Lac; n = 29; **B**); the Y-chromosome (CEN-Y; n = 18; **D**); and neocentric chromosome 4 (NeoCEN-4; n = 39; **F**) of indicated cell lines compared to all other centromeres within the same cell (Other CENs; n = 1008, 620, and 1592, respectively). Median (line), interquartile distance (box), 3 × interquartile distance or extremes (whiskers), and outliers (spots) are indicated. ***Figure 7—figure supplement 1*** shows results of individual centromeres. Asterisk indicates statistically significant difference (*t* test; $p<0.05$); NS indicates no significant difference. (**G**) Representative images of CENP-A antibody staining in indicated cell types. Images of RPE cells are shown as independent reference. Primary fibrobl. indicates primary human foreskin fibroblasts. (**H**) Quantification of **G**. Mean ± SEM for n = 3–4 independent experiments is shown. Left y-axis represents centromeric CENP-A levels normalized to RPE cells; right y-axis shows number of CENP-A molecules per centromere (CEN). (**I**) Combined results from **A**–**H** allow for the determination of CENP-A copy numbers on individual chromosomes as indicated. (**J**) Statistical map of the distribution of 216 CENP-A nucleosomes on the NeoCEN-4 at three different scales. The top 216 peaks are indicated in blue. Y-axis indicates the probability of CENP-A occupancy for each nucleosome. (**K**) Histogram of the CENP-A nucleosome occupancy. Inset shows the distribution of 216 neocentric CENP-A nucleosomes on the 10% highest occupancy peaks (green) and 90% lowest occupancy peaks (red).

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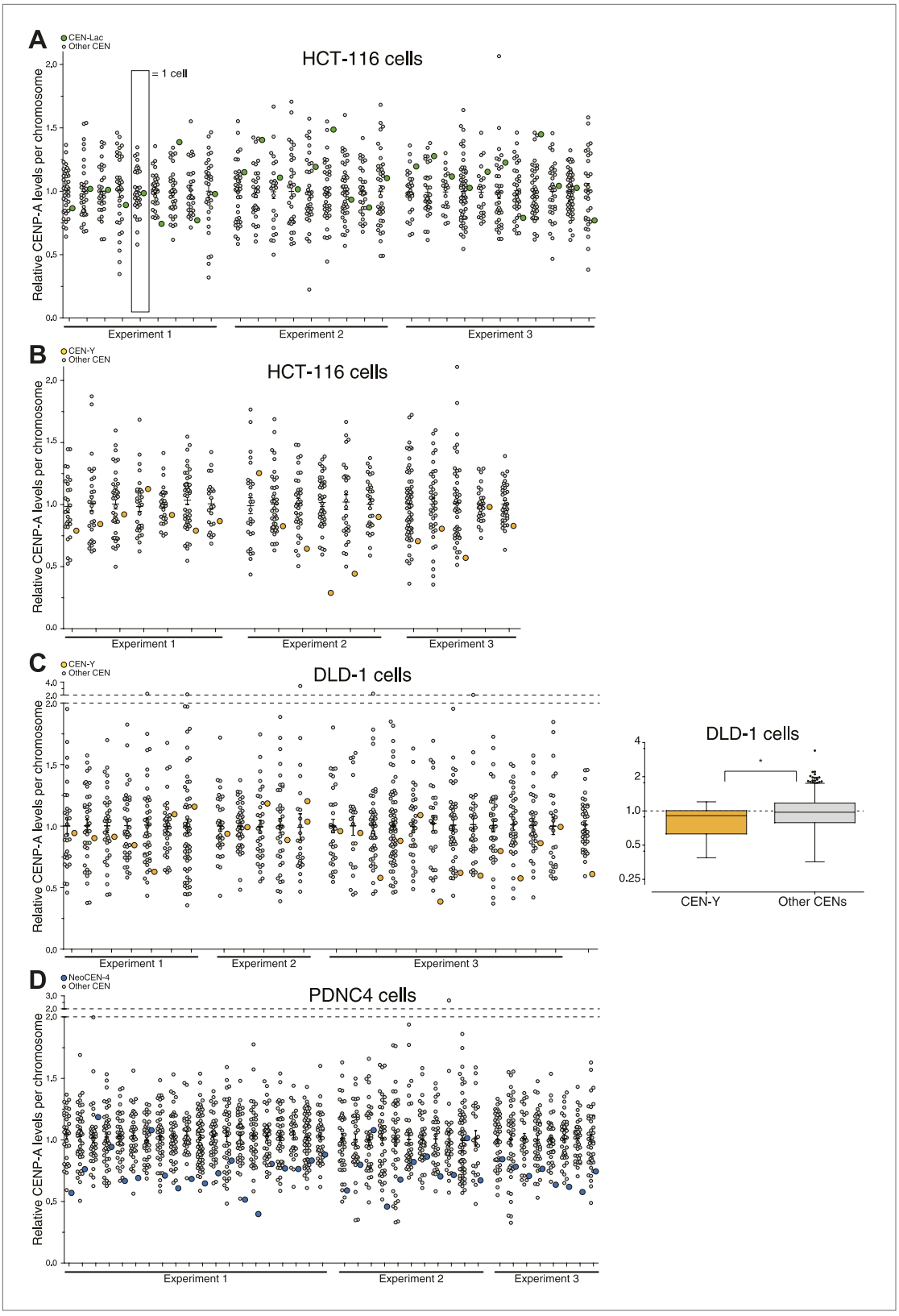

Figure 7—figure supplement 1. Measurements of individual centromeres for graphs in ***Figure 7A–F***.

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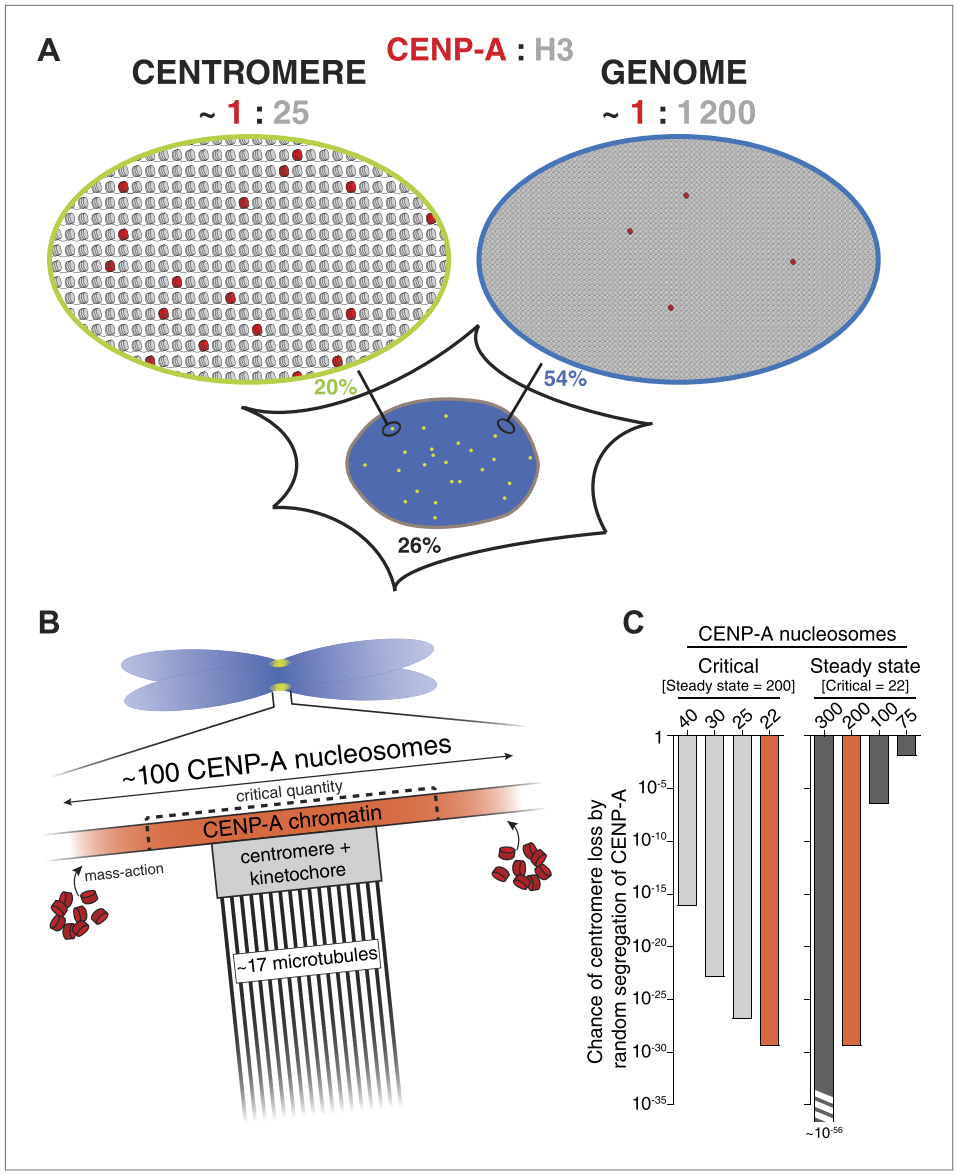

Figure 8. A quantitative view of human centromeric chromatin. (**A**) Distribution of CENP-A. Estimated ratio of CENP-A (red) to H3 (gray) at the centromere and on non-centromeric loci (genome) in interphase cells. Estimations are calculated assuming 2 CENP-A molecules per nucleosome (***Sekulic et al., 2010***; ***Tachiwana et al., 2011***; ***Bassett et al., 2012***; ***Hasson et al., 2013***; ***Padeganeh et al., 2013***), an average nucleosome positioning distance of 200 base pairs, an average centromere size of 2.5 × 10^6 base pairs (***Sullivan et al., 1996***; ***Lee et al., 1997***) of which approximately 40% (1 Mbp) contains CENP-A (***Sullivan et al., 2011***), a diploid genome size of 6 × 10^9 base pairs, 200 CENP-A nucleosomes per centromere, and 2.5 × 10^4 CENP-A nucleosomes outside of centromeres (9.1 × 10^4 CENP-A molecules per cell [***Figure 2F***], of which 74% is in chromatin [***Figure 4B***] and 0.44% in each centromere [***Figure 2B***]). The fraction of CENP-A on centromeres, non-centromeric chromatin, and unincorporated CENP-A are indicated in green, blue, and black, respectively. CENP-A nucleosomes are represented as though evenly spread throughout the centromeric domain. Alternatively, they could be distributed into one or more clusters within this domain. (**B**) Mitotic organization of centromeric chromatin. 200 nucleosomes are redistributed to 100 nucleosomes per centromere on replicated sister chromatids (***Jansen et al., 2007***; ***Bodor et al., 2013***). The exact CENP-A copy number at the centromere depends on the available total pool (mass-action). Excess CENP-A could either lead to an increased CENP-A domain or lead to a higher density of CENP-A within a domain of fixed size. This pool forms an excess to recruit downstream centromere and kinetochore complexes and ultimately provides microtubule binding sites for ~17 kinetochore microtubules (***McEwen et al., 2001***). To avoid mitotic errors, a critical amount of CENP-A is required (dashed lines). (**C**) Graph representing the chance of at least one

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chromosome in a cell (with 46 chromosomes) reaching critically low levels of CENP-A by random segregation of pre-existing CENP-A nucleosomes. Calculations were performed for varying levels of critical nucleosome numbers at a fixed steady state of 200 (left), or by varying the steady state number at a fixed critical level of 22 (right). Red bars represent identical calculations.

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